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THE DETECTION OF PATHOGENIC *LEPTOSPIRA* SPECIES USING A POLYMERASE CHAIN REACTION TEST

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Abstract

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Pathogenic *Leptospira* species cause leptospirosis in animals and humans. Bacterial culture and the serological identification of leptospires are constrined by being time consuming and labor intensive. Oligonucleotide primers complementary to the 16S ribosomal RNA gene sequence of pathogenic leptospires were designed to amplify the 343 bp fragment. The primers were specific to pathogenic Leptospira species and did not amplify either non-pathogenic leptospires or common bacteria found in urine. This developed PCR assay allows the rapid identification of pathogenic leptospires from rodent urine, with high sensitivity and specificity.

Keywords : *Leptospira* spp., Leptospirosis, Polymerase Chain Reaction

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บทคัดย่อ

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การตรวจพิสูจน์เชื้อเลปโตสไปร่า โดยวิธีปฏิกริยาลูกโซ่โพลิเมอเรส

เชื้อเลปโตสไปร่า เป็นเชื้อแบคทีเรียที่ทำให้เกิดโรคเลปโตสไปโรซีสทั้งในคนและสัตว์ การตรวจพิสูจน์เชื้อโดยวิธีเพาะเลี้ยงเชื้อและวิธีซีรั่มวิทยา มีข้อจำกัดเนื่องจากใช้เวลานานและมีขั้นตอนที่ยุ่งยาก การศึกษาครั้งนี้ได้ออกแบบ primers ที่มีความจำเพาะต่อยีน 16S ribosomal RNA ของเชื้อเลปโตสไปร่าด้วยการเพิ่มจำนวนดีเอ็นเอในขนาด 343 คู่เบส ผลการศึกษาพบว่าวิธีปฏิกิริยาลูกโซ่โพลิเมอเรสนี้ มีความจำเพาะต่อเชื้อเลปโตสไปร่าที่ทำให้เกิดโรค แต่ไม่ให้ผลบวกกับเชื้อเลปโตสไปร่าที่ไม่ก่อให้เกิดโรคและเชื้อแบคทีเรียชนิดอื่นๆ ที่พบได้ในปัสสาวะ วิธีปฏิกริยาลูกโซ่โพลิเมอเรสที่พัฒนาขึ้นมานี้ยังสามารถนำมาใช้ในการตรวจพิสูจน์เชื้อเลปโตสไปร่าในปัสสาวะจากหนูได้อย่างรวดเร็ว รวมทั้งมีความไวและความจำเพาะสูง

คำสำคัญ : เชื้อเลปโตสไปร่า โรคเลปโตสไปโรซีส ปฏิกิริยาลูกโซ่โพลิเมอเรส

Introduction

Leptospirosis is a widespread zoonotic disease caused by pathogenic *Leptospira* species. The disease occurs in wild and domesticated animals as well as in humans. Infection with pathogenic leptospires causes subclinical or chronic symptoms depending on the *Leptospira* serovar and the host species (Alexander, 1974; Faine et al., 1999). Detection of *Leptospira* species is usually based on conventional culture methods, serological methods, such as the microscopic agglutination test (MAT), the enzyme-linked immunosorbent assay (ELISA), and the DNA hybridization methods (Brandao et al., 1998; Faine et al., 1999; Trueba et al., 1990).

Currently, there are some limitations in the detection of leptospires from both humans and animals. Conventional bacterial culture may take upto 12 weeks and requires a special medium (Bolin et al., 1989). The MAT is already standardized and can be used to detect anti-leptospiral antibodies, however, cross-reactions of serovar specific antibodies can often be observed. In addition, the MAT is labor intensive, expensive and is not a good rapid screening test (Cumberland et al., 1999). The ELISA is more sensitive than the MAT but the technique has limitations regarding the standardization of infectious and non-infectious antibody titers, especially in animals (Wagenaar et al., 1994). Probe hybridization for the detection of leptospires has been reported but the technique has the major drawback of low sensitivity (McCormick et al., 1989; Zuerner and Bolin, 1988).

The use of the polymerase chain reaction test (PCR) for the detection of leptospires has been previously documented. The PCR amplifications can be based on specific target sequences including the 16S ribosomal RNA (rRNA) gene (Faber et al., 2000; Merien et al., 1992; Romero et al., 1998), the 23S rRNA gene (Woo et al., 1997), insertion sequences (Zuerner and Bolin, 1997), repetitive elements (Savio et al., 1994), and the endoflagellin gene (Woodward and Redstone, 1993). In addition, some investigators also reported the use of the PCR to detect and discriminate between pathogenic and non-pathogenic leptospires (Letocart et al., 1997; Wagenaar et al., 1994). In this study, we developed a simple and rapid PCR assay for identifying pathogenic leptospires found in animal urine.

Materials and methods

Bacterial isolates. Thirty *Leptospira* strains and 15 uropathogenic bacteria were used to test the PCR assay (Table 1). *Leptospira* strains were obtained from the culture collection of the Armed Force Institute of Medical Researches (AFIMRS), Bangkok, Thailand. On receipt, all Leptospira strains were subcultured onto Feltcher medium (Difco Laboratories, Detroit, MI) at 25°C fo up to 12 weeks and were confirmed to be *Leptospira* on the basis of culture and serological characteristics. Fifteen uropathogenic bacteria were provided by the Department of Microbiology, Faculty of Medicine and Veterinary Sciences, Chulalongkorn University and were subcultured onto Nutrient agar (Difco Laboratories) at 37°C for 24 hrs. Freezer stocks of bacterial isolates were prepared and stored in a liquid medium with 20% glycerol, either at -80°C or in liquid nitrogen, until further use.

Chromosomal DNA extraction. The methods used to prepare chromosomal DNA from the bacterial isolates have been described previously (Amonsin et al., 1997). The DNA was resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA [pH 8.0]) and was quantified by both gel electrophoresis and spectrophotometry (Shimadzu Corporation, Kyoto, Japan).

DNA amplification by PCR. The alignment of the 16S rRNA gene sequences of the *Leptospira* species was performed using the MegAlign program (DNASTAR Inc, Madison, WI). DNA sequences were obtained from GenBank database and included *L. borgpetersenii* serovar hardjo strain Hardjo-bovis (accession number U12670), *L. inadai* serovar inadai strain Lyme (accession number Z21634), *L. interrogans* serovar icterohaemorrhagiae strain RGA (accession number Z12817), *L. kirschneri* serovar cynopteri strain 3522 C (accession number Z21628), *L. nogucii* serovar panama strain CZ 214 K (accession number Z21635), *L. santarosai* serovar shermani strain 1342 K (accession number Z21649), *L. weilli* serovar celledoni strain Celledoni (accession number Z21637), *L. biflexa* serovar patoc strain PatocI (accession number 21821) and *L. meyeri* serovar semaranga strain Veldrat semarang 173 (accession number AF167353). Conserved regions of the pathogenic leptospires were identified and oligonucleotide primers were designed based on these particular regions. In each PCR reaction, 1 ng of purified DNA or 2 µl of boiled cell lysate was used as a DNA template. The 20 µl PCR reaction mixture was made up in PCR buffer II (Fermentas, Hanover, MD) and contained 200 pmol of each primer; 200 µM of each dNTP; 2.5 mM $MgCl_2$; and 0.5 U of AmpliTaq DNA polymerase (Fermentas). The PCR reaction was carried out in a thermal cycler (Hybaid Limited, Ashford, Middlesex, UK) with initial denaturation at 94°C for 3 min, followed by 30 PCR cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. A 7 µl volume of PCR products was mixed with 2 µl of loading buffer (0.2% Orange G in 50% glycerol), and the mixture was electrophoresed in 1.5% agarose (FMC Bioproducts, Rockland, ME) with 0.5 mg of ethidium bromide per ml. Agarose gel was photographed under UV light with a gel documentation system (Vilber Lourmat, La Valle Cedex, France). To confirm the specificity of the PCR, the amplified product from *L. icterohaemorrhagiae* ATCC 43642 was sequenced and compared with the 16S rRNA gene sequence in GenBank (accession number Z12817). To confirm the sensitivity of the technique, serial 10-fold dilutions of *L. icterohaemorrhagiae* ATCC 43642 were used to seed the urine samples and examine the sensitivity of the PCR assay. To confirm the reproducibility of the PCR assay, two separated reactions were conducted for each isolate.

Urine samples. Urine samples were collected from 245 rodents trapped in Kanchanaburi province, Thailand. The animals were euthanased and 200-500 µl of urine samples were collected by an aseptic technique. If the urine sample was less than 200 µl, 1000 µl of sterile phosphate buffer saline was added to flush out the urine.

Preparation of urine samples for PCR. The method used to prepare the urine samples for PCR was a modification of one described previously (Bal et al., 1994; Merien et al., 1992). 500 µl of 1M NaCl was added to each 500 µl of urine. The sample was then centrifuged for 15 min at 13,000 x g. The pellet was resuspended in 100 µl of 1 mM EDTA (pH 8.0) and was centrifuged for 15 min at 13,000 x g. The pellet was then resuspended in 10 µl of TE. The mixture was heated for 15 min at 100°C before being used as a DNA template for the PCR reaction.

Table 1. A list of the *Leptospira* strains and the uropathogenic bacteria used in this study.

Species	Serogroup	Serovar	Strain	PCR
L. biflexa	Semanranga	patoc	Patoc I	-
L. biflexa	Unknown	P 136	P 136	-
L. biflexa	Unknown	P 138	P 138	-
L. borgpetersenii	Ballum	ballum	Mus 127	+
L. borgpetersenii	Javanica	javanica	Veldrat Batavia 46	+
L. borgpetersenii	Javanica	poi	Poi	+
L. borgpetersenii	Sejroe	sejroe	M 84	+
L. borgpetersenii	Tarassovi	tarassovi	Mitis Johnson	+
L. interrogans	Australis	australis	Unknown	+
L. interrogans	Australis	australis	Ballico	+
L. interrogans	Australis	bangkok	Bangkok D92	+
L. interrogans	Australis	bratislava	Jez Bratislava	+
L. interrogans	Autumnalis	autumnalis	Unknown	+
L. interrogans	Autumnalis	akiyami A	Akiyami A	+
L. interrogans	Autumnalis	rachmati	Rachmat	+
L. interrogans	Bataviae	bataviae	Swart	+
L. interrogans	Cannicola	cannicola	Hond Utrecht IV	+
L. interrogans	Djasiman	djasiman	Djasiman	+
L. interrogans	Grippotyphosa	grippotyphosa	Moskva V	+
L. interrogans	Hebdomadis	hebdomadis	Hebdomadis	+
L. interrogans	Icterohaemorrhagiae	copenhageni	M 20	+
L. interrogans	Icterohaemorrhagiae	icterohaemorrhagiae	RGA (ATCC 43642)	+
L. interrogans	Pomona	pomona	Pomona	+
L. interrogans	Pyrogenes	pyrogenes	Salinem	+
L. interrogans	Sejroe	hardjo	Hardjoprajitno	+
L. interrogans	Sejroe	wolffii	Wolffii	+
L. kirschneri	Cynopteri	cynopteri	3522 C	+
L. weilii	Celledoni	celledoni	Celledoni	+
Uncertain	Autumnalis	new	Heusden p2062	+
Uncertain	Lousiana	Saigon	L 79	+
Enterobacter cloacae			DMST3557[a]	-
Enterococcus faecalis			ATCC33186[b]	-
Escherichia coli			ATCC 35218	-
Proteus mirabilis			DMST 8211	-
Proteus vulgaris			DMST 0557	-
Pseudomonas aeroginosa			ATCC 27853	-
Serratia marcescens			ATCC 8100	-
Staphylococcus aureus			ATCC 12715	-
Staphylococcus epidermidis			ATCC 12228	-
Staphylococcus hyicus			DMST 4008	-
Staphylococcus intermedius			DMST 3784	-
Staphylococcus saprophyticus			DMST 3558	-
Alcaligenes faecalis			ATCC 35655	-
Klebsiella pneumoniae			ATCC 27736	-
Streptococcus milleri			DMST 4956	-

[a] DMST: Department of Microbiology, Chulalongkorn University Hospital, Bangkok, Thailand

[b] ATCC: American Type Culture Collection

Results

An alignment of the 16S rRNA gene sequences was performed and oligonucleotide primers were designed, as shown in figure 1. Oligonucleotide primers, designated Lepto-F, 5'- TCYGAGTCTGGGATAACTTTCC and Lepto-R 5'-GTACCATCATCACATYGCTG (Y; T or C), were synthesized and obtained from GibcoBRL (Life Technologies, Frederick, MD). The expected size of the amplified product was 343 bp. PCR amplification of purified DNA from 27 pathogenic leptospires using oligonucleotide primers, Lepto-F and Lepto-R, generated a DNA fragment of the expected 343 bp PCR product. In addition, PCR products were not observed in the 3 non-pathogenic *Leptospira* strains and all the other uropathogenic bacteria that were tested (Table 1 and Fig 2). Sequence analysis of the 343 bp amplified product confirmed the identity of the 16S rRNA fragment of *L. icterohaemorrhagiae* ATCC 43642 (Fig 3). The sensitivity of the PCR assay, which was examined in ten-fold serial dilutions of bacterial cultures, seeded in urine samples, was 50 leptospires/ml of urine (Fig 4). This PCR assay gave reproducible and reliable results in the two separated PCR reactions which were performed on each bacterial strain.

In order to test the application of the developed PCR assay, we used the PCR assay to detect pathogenic leptospires in urine samples collected from rodents in a rural area of Thailand, Kanchanaburi Province. Only 2.04% (5/245) of urine samples were positive for pathogenic leptospires. Out of the five positive urine samples, three were positive by culture technique (data not shown).

Discussion

This study describes the development of a PCR assay for the rapid identification of pathogenic *Leptospira* species from animal urine. In order to develop PCR assay, an alignment of the 16S rRNA gene sequences of pathogenic and non-pathogenic leptospires was carried out (Fig 1). In this study, the 16S rRNA gene sequence was selected as a target region because it is relatively conserved in most bacterial species. The amplification of this gene may therefore be useful for the identification of and the discrimination between, pathogenic and non-pathogenic leptospires (Wagenaar et al., 1994). The amplification of *Leptospira* DNA, based on it's 16S rRNA gene sequences, have been previously reported (Faber et al., 2000; Merien et al., 1992; Romero et al., 1998; Wagenaar et al., 1994). However, only a few papers reported the detection and differentiation of pathogenic and non-pathogenic leptospires based on such sequences. In this study, the nucleotide sequences of the forward primer (position 69-91) and the reverse primer (392-411) were targeted for pathogenic leptospira and somewhat different from non-pathogenic leptospira (Fig 1). As a result this primer set can differentiate between pathogenic and non-pathogenic leptospires.

The oligonucleotide primers described in this study amplified the 343 bp fragment of the 16S rRNA gene sequence and is unique for pathogenic *Leptospira* species. The results showed that the PCR assay had high sensitivity and specificity. The assay has high specificity, because all 27 pathogenic *Leptospira* species could be detected by the assay. On the other hand, non-pathogenic leptospires and other uropathogenic bacterial species could not be detected (Table 1). The specificity of the PCR assay was also confirmed by sequence analysis of the 343 bp amplified products. The result of the DNA sequence analysis of 288 nucleotides of the PCR product showed 100% match to *L. icterohaemorrhagiae* ATCC 43642. We found that the DNA sequencing and sequence analysis provided rapid and accurate data for confirming the PCR amplicon. Currently, the DNA sequence analysis is more commonly used to confirm the amplified PCR product than probe hybridization (Jiang et al., 2000). The sensitivity of the PCR assay was examined using 10-fold serial dilutions of *L. icterohaemmorhagiae* ATCC 43642 seeded in urine samples. The sensitivity of the PCR assay was 50 leptospires/ml of urine (Fig 4). This sensitivity is similar to the sensitivity of the PCR assay as described by others which ranged from 10-1000 bacterial cells/ml (Heinemann et al., 2000; Wagenaar et al., 1994).

Five pathogenic leptospires were detected from 245 samples (2.04%) of rodent urine collected from a rural area of Thailand. The result showed that a developed PCR assay can be used for the rapid identification of pathogenic leptospires from such urine samples. The rapid identification of pathogenic leptospires is essential in order to identify the reservoirs and animal sources associated with human infections. Rapid *Leptospira* identification using the PCR can help investigators to recognize the sources of infection in leptospirosis outbreaks and is useful than the serological test, which is labor intensive and slow. However, the disadvantage of PCR assay is its inability to assign a Leptospira serovar. To overcome this limitation, we suggest the use of DNA fingerprinting techniques to characterize the leptospires. The information gained from DNA fingerprinting can be used to identify the sources of infection while investigating a Leptospirosis outbreak *Leptospira* is commonly found in rodents such as rats *(Rattus norvegicus)* and mice *(Mus musculus)*, which are reservoirs of leptospires. In this study, we found that 2.04% of rodents carried pathogenic leptospires in their urine. The prevalence of pathogenic leptospires in rodents in Thailand has been rarely reported. The prevalence of leptospires in rodents may vary depending on the animal habitats, types of samples and the number of organisms present in the animals (Faine et al., 1999).

In conclusion, a PCR assay has been developed for the detection of pathogenic leptospires in rodent urine. The assay provides for rapid identification of leptospires with high sensitivity and specificity. The prevalence of pathogenic leptospires recovered from rodent urine in Thailand has also been investigated.

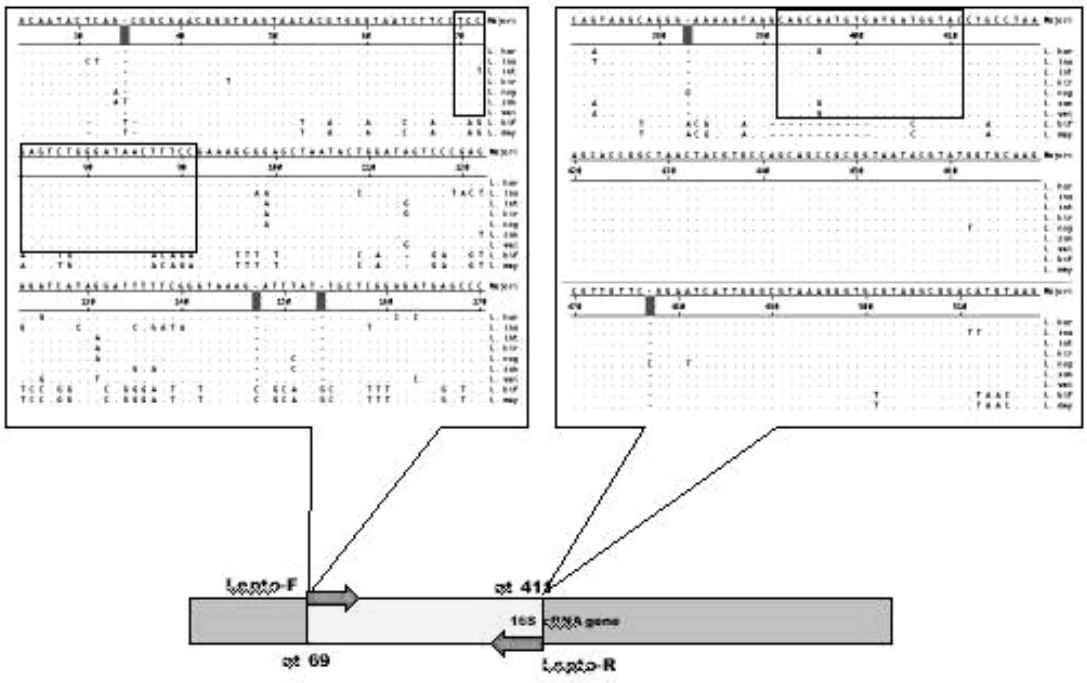

Figure 1. A schematic representation of the alignment of the 16S rRNA gene sequences of pathogenic and non-pathogenic leptospires. Boxes show the primers designed from the variable sequences that differentiate between the pathogenic and the non-pathogenic leptospires. The primer sequences (Lepto-F and Lepto-R) are shown. L. har = *L. borgpetersenii* serovar hardjo strain hardjo-bovis; L. ina = *L. inadai* serovar inadai strain Lyme; L. int = *L. interrogans* serovar icterohaemorrhagiae strain RGA; L. kir = *L. kirschneri* serovar cynopteri strain 3522; L. nog = *L. nogucii* serovar panama strain CZ 214 K; L. san = *L. santarosai* serovar shermani strain 1342K; L. wei = *L. weilli* serovar celledoni strain Celledoni; L. bif = *L. biflexa* serovar patoc strain Patoc I; L mey = *L. meyer*i serovar semaranga strain Veldrat semarand 173.

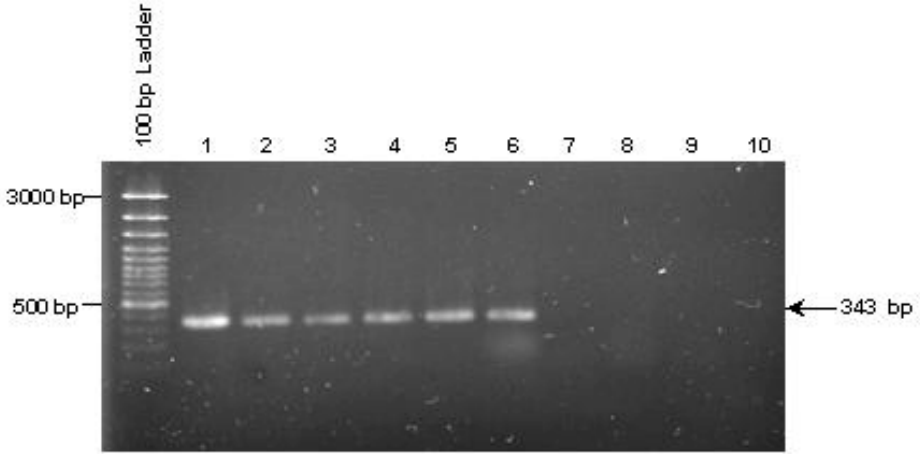

Figure 2. The PCR amplification products generated by Lepto-F and Lepto-R primers. Lane 1-6 pathogenic *Leptospira* species; *L. hardjo, L. bratislava, L. icterohaemorrhagiae, L. canicola, L. bangkok, L. ballum*, respectively. Lane 7-9 non-pathogenic *Leptospira* species; *L. patoc*, *Leptospira* P136, *Leptospira* P138, Lane 10, Negative control. The arrow indicates the 343 bp amplified products.

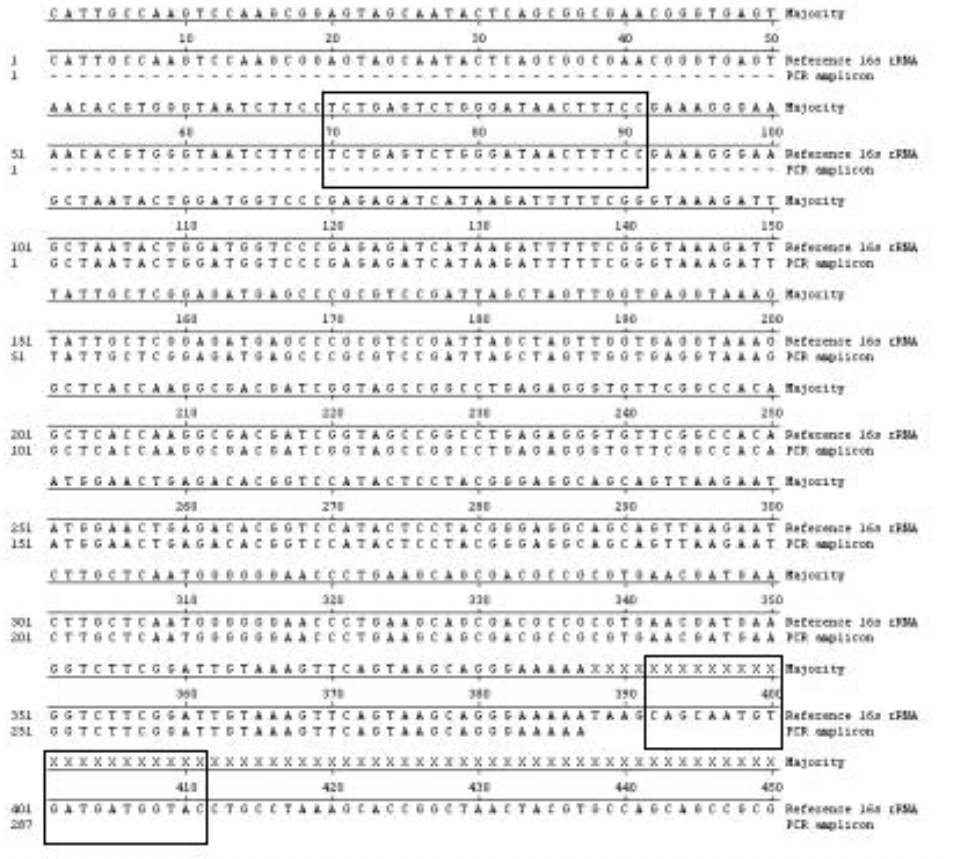

Figure 3. The alignment of the *L. icterohaemorrhagiae* ATCC 43642 16S rRNA gene sequence and the 288 bp PCR amplicon (amplicon size, 343 bp). The 16S rRNA gene sequence and the PCR amplicon are shown at the top and bottom, respectively. The boxes represent forward and reverse primers.

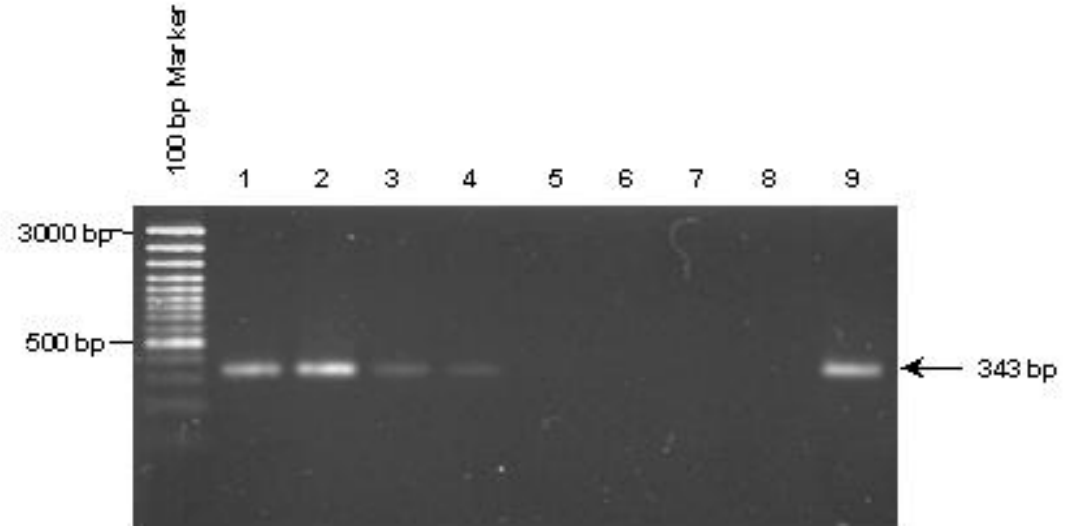

Figure 4. The sensitivity of the PCR assay. Lane 1-7 *L. icterohemorrhagiae* ATCC 43642 in ten-fold serial dilutions; $5x10^4$, $5x10^3$, $5x10^2$, 50, 5, 0.5, 0.05 leptospires/ml of urine. Lane 8, Negative control. Lane 9, Positive control. The arrow indicates 343 bp amplified products.

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